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Supplemental Assay Method for Immunogenicity and Spore Count Testing of Anthrax Spore Vaccine-Nonencapsulated

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Supplemental Assay Method for Immunogenicity and Spore Count Testing of Anthrax Spore Vaccine-Nonencapsulated

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1. Introduction

This Supplemental Assay Method (SAM) describes methods for immunogenicity and spore count testing of Anthrax Spore Vaccine-Nonencapsulated (ASV-N) as prescribed in the Code of Federal Regulations, Title 9 (9 CFR), Part 113.66. **Part I** describes the guinea pig vaccination-challenge test used to determine immunogenicity of ASV-N Master Seed. **Part II** describes the spore count procedure for release testing of final container samples of ASV-N.

Part I. Immunogenicity Test

2. Materials

2.1 Equipment (Unless specified, equipment vendors are optional.)

2.1.1 Class II biological safety cabinet

2.1.2 Autoclave

2.1.3 Pipette filler

2.2 Reagents/supplies

2.2.1 *Bacillus anthracis* challenge culture, IRP 137 (This culture must be obtained from the United States Department of Agriculture, Animal and Plant Health Inspection Service, Veterinary Services, Center for Veterinary Biologics [CVB], Ames, IA 50010.)

2.2.2 Glycerol diluent

2.2.3 Disposable syringes, 3-ml and 5-ml

2.2.4 Needles, 18-gauge x 1 1/2-inch and 23-gauge x 1-inch

2.2.5 Glass pipettes, 2-ml and 5-ml

2.2.6 Glass volumetric pipettes, 100-ml or 25-ml

2.2.7 Glass screw-top Erlenmeyer flask, 500-ml, with cap

2.2.8 Glass screw-top tubes, 16 x 125-mm, with caps

2.2.9 Glass screw-top dilution bottles, 160-ml

2.2.10 Glass serum bottles, 100-ml

2.2.11 Rubber seals and metal caps for serum bottles

2.2.12 Disposable clothing

2.2.13 Occupational Safety and Health Administration (OSHA)-approved dust and mist respirator

2.3 Test animals

Guinea pigs, 400-500 g. Forty-two guinea pigs (30 vaccinates and 12 controls) from the same source are required for each lot of Master Seed to be tested. (Guinea pigs of either sex or any color may be used.)

3. Preparation for the immunogenicity test

3.1 Personnel qualifications/training

Technical personnel need a working knowledge of the use of general laboratory chemicals, equipment, and glassware. In addition, personnel need specific training and experience in the safe handling of live *B. anthracis* cultures.

3.2 Preparation of supplies

3.2.1 Sterilize all glassware before use.

3.2.2 Make sure all disposable supplies (pipettes, syringes, needles, rubber stoppers, etc.) are sterile.

3.3 Handling of test animals

3.3.1 Select guinea pigs that appear healthy, are free of external parasites, and have an unblemished hair coat.

3.3.2 Examine and cage the guinea pigs the day they are received. (Up to 4 guinea pigs of the same sex may be housed in each cage.)

3.3.3 House the test animals in a room with access limited to authorized personnel only.

3.3.4 Wear disposable outer clothing and an OSHA-approved dust and mist respirator for protection during and after vaccination and challenge. Use the disposable clothing and respirator only once and discard them before exiting the animal room.

3.3.5 Euthanize and incinerate the guinea pigs when the test is concluded. Disinfect the contaminated equipment and cages. Sterilize the bedding before disposal.

3.4 Preparation of reagents

3.4.1 *B. anthracis* challenge culture

The challenge culture, *B. anthracis* IRP 137, is suspended in 50% glycerol. Each ampule contains approximately 1.8 ml of culture suspension. Store ampules of challenge at -70°± 5°C.

3.4.2 Glycerol diluent, 50%

The 50% glycerol diluent is prepared by mixing equal parts of glycerol and 0.85% NaCl solution. Place 300 ml of diluent in a 500-ml flask and sterilize at 121°C for 25-30 minutes. Store at room temperature up to 1 year.

4. Performance of the immunogenicity test

4.1 Vaccination of test animals

4.1.1 Check the label on each container of Master Seed to be tested for identity.

4.1.2 Thoroughly mix the Master Seed by striking the container against the palm of the hand at least 25 times before filling the syringes. Use 3-ml or 5-ml syringes, fitted with 23-gauge x 1-inch needles.

4.1.3 Vaccinate 30 guinea pigs with the required dosage of the Master Seed as recommended on the label. [See 9 CFR 113.66(b)(2).]

4.1.4 Vaccinate each animal only once.

4.1.5 Observe the guinea pigs for 14-15 days. Examine each animal carefully for sores, edema, and general physical condition. Record deaths.

4.2 Timing of challenge

4.2.1 Challenge vaccinated guinea pigs 14-15 days after vaccination.

4.2.2 Challenge nonvaccinated control guinea pigs at the same time.

4.3 Preparation of challenge dilutions

Caution: The *B. anthracis* challenge is a live spore suspension. Prepare the challenge within a Class II biological safety cabinet. Use a pipette filler. DO NOT MOUTH PIPETTE LIVE CULTURE. Autoclave any contaminated equipment and clothing before disposal.

4.3.1 Warm an ampule of challenge culture suspension to room temperature.

4.3.2 Mix the contents thoroughly by shaking the ampule vigorously.

4.3.3 Disinfect the ampule with 70% ethyl alcohol. Carefully break open the ampule using a pledget of dry, sterile cotton to enclose the break line.

4.3.4 Use a 3-ml syringe fitted with an 18-gauge x 1 1/2-inch needle to aseptically transfer the culture suspension to a 16 x 125-mm test tube. Thoroughly mix the suspension.

4.3.5 Place 4 ml of glycerol diluent into a 16 x 125-mm test tube using a sterile 5-ml pipette. Use a sterile 2-ml pipette to add 1 ml of culture suspension to the 4 ml of glycerol diluent. **(Note: The 50% glycerol is viscid and must be pipetted with**

care to insure delivery of the complete volume.) Mix thoroughly.

4.3.6 Measure 100 ml of glycerol diluent into a 160-ml glass dilution bottle using a 100-ml or 25-ml volumetric pipette. Remove 1 ml of glycerol diluent using a 2-ml pipette thereby leaving 99 ml of diluent in the dilution bottle. Using a 2-ml pipette, transfer 1 ml of the 1:5 challenge dilution to the 99 ml of glycerol diluent. Mix thoroughly. (This is the 1:500 dilution.)

4.3.7 Measure 100 ml of glycerol diluent into a 100-ml serum vial using a 100-ml or 25-ml volumetric pipette. Remove 1 ml of glycerol diluent using a 2-ml pipette thereby leaving 99 ml of diluent in the serum vial. Using a 2-ml pipette, transfer 1 ml of the 1:500 challenge dilution to the 99 ml of glycerol diluent. Cap and seal the serum vial. Mix thoroughly. (This is the 1:50,000 challenge dilution.) The challenge material is kept at room temperature until it is used to inoculate the guinea pigs.

4.4 Inoculation of guinea pigs

4.4.1 Thoroughly mix the 1:50,000 challenge dilution before filling syringes. (Use 3-ml syringes fitted with 23-gauge x 1-inch needles.)

4.4.2 Inoculate 0.5 ml of the challenge into the thigh muscle of each guinea pig. (This inoculum contains approximately 4500 guinea pig lethal dose 50 [LD_{50}] of spores.)

4.5 Observation of guinea pigs following challenge

4.5.1 Observe the guinea pigs daily for 10 days. Examine the test animals for sores, edema, and general physical condition. Record the observations on the daily record. Record the number of deaths.

Note: Moribund animals exhibiting clinical signs consistent with the expected disease pathogenesis that are unable to rise or move under their own power may be humanely euthanized and considered as deaths as outlined in 9 CFR 117.4.

5. Interpretation of the immunogenicity test results

5.1 The test is interpreted as prescribed in 9 CFR, Part 113.66.

5.1.1 If at least 10 of the 12 controls die from *B. anthracis* within the 10-day postchallenge observation period, the test is valid. If 9 or fewer of the 12 controls die from *B. anthracis* within the 10-day observation period, the test is invalid and may be repeated.

5.1.2 If 27 or more of the 30 vaccinates survive the 10-day observation period, the Master Seed is satisfactory.

5.1.3 If not at least 27 of the 30 vaccinates survive the 10-day observation period, the Master Seed is unsatisfactory.

5.2 Observation of sores, edema, and general physical condition provides supplemental information concerning the safety and quality of the Master Seed. However, potency test results are evaluated only on survival/death patterns.

6. Reporting of the immunogenicity test results

Report results of the test(s) as described by standard Section operating procedures.

Part II. ASV Spore Count Test

2. Materials

2.1 Equipment (Unless specified, equipment vendors are optional.)

2.1.1 Class II biological safety cabinet

2.1.2 Autoclave

2.1.3 Water bath, set at 50°C

2.1.4 Incubator, set at 37°C

2.1.5 Colony counter

2.1.6 Pipette filler

2.2 Reagents/supplies

2.2.1 Reference vaccine

2.2.2 Glycerol diluent

2.2.3 Tryptose agar

2.2.4 Glass test tubes, 20 x 150-mm, with Morton closures

2.2.5 Glass screw-top test tubes, 20 x 150-mm, with caps

2.2.6 Glass screw-top test tubes, 16 x 125-mm, with caps

2.2.7 Needles, 18-gauge x 1- or 1 1/2-inch

2.2.8 Glass screw-top dilution bottles, 160-ml, with caps

2.2.9 Glass pipettes, 2-ml, 5-ml, and 10-ml

2.2.10 Petri dishes, 100 x 15-mm

2.2.11 Disposable syringes, 3-ml

2.2.12 Glass volumetric pipettes, 100-ml or 25-ml

3. Preparation for the spore count test

3.1 Personnel qualifications/training

Technical personnel need a working knowledge of the use of general laboratory chemicals, equipment, and glassware. In addition, personnel need specific training and experience in the safe handling of live *B. anthracis* cultures.

3.2 Preparation of supplies

3.2.1 Sterilize all glassware before use.

3.2.2 Make sure all disposable supplies (pipettes, syringes, needles, rubber stoppers, etc.) are sterile.

3.3 Preparation of reagents

Caution: The Anthrax Spore Vaccine-Nonencapsulated is a live spore suspension. Prepare the reference and serial vaccine dilutions within a Class II biological safety cabinet. Use a pipette filler. DO NOT MOUTH PIPETTE LIVE VACCINE. Autoclave any contaminated equipment and clothing before disposal or reuse.

3.3.1 Use a reference vaccine to be tested with the test vaccine. (The reference vaccine serves as the control for the media and the technique. The reference should have been shown to produce consistent spore counts in at least 3 previous tests.)

3.3.2 Prepare the 50% glycerol diluent by mixing equal parts of glycerol and 0.85% NaCl solution. Place 300 ml of diluent in a 500-ml flask. Autoclave at 121°C for 25-30 minutes. Store at room temperature up to 1 year.

3.3.3 Prepare tryptose agar according to label directions on the day of the test. Dispense tryptose agar in 20 ml amounts in 20 x 150-mm test tubes with Morton closures. Autoclave 25-30 minutes at 121°C.

4. Performance of the spore count test

4.1 Adjust water bath to 50°± 5°C.

4.2 Prepare tryptose agar as described above in **Part II, Section 3.3.3.** Cool tryptose medium in the water bath approximately 1 hour.

4.3 Vigorously shake each vaccine by hand 100 times before making dilutions.

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4.4 Measure 100 ml of glycerol diluent into each of two 160-ml dilution bottles using a 100-ml or 25-ml volumetric pipette. Remove 1 ml of glycerol diluent from each bottle using a 2-ml pipette thereby leaving 99 ml of glycerol diluent in each bottle.

4.5 Remove 2-3 ml of vaccine with a 3-ml syringe and 18-gauge x 1 1/2-inch needle and transfer to a sterile 16 x 125-mm glass screw cap test tube. Use a 2-ml pipette to transfer 1 ml of vaccine to 99 ml of glycerol diluent in the dilution bottle. This 1:100 dilution approximates 15,000 spores/ml in a 2-ml vaccine dose and 30,000 spores/ml in a 1-ml vaccine dose.

4.6 Vigorously shake the 1:100 dilution by hand 100 times. Make another hundredfold dilution by transferring 1 ml of the 1:100 dilution into 99 ml of glycerol diluent in the next dilution bottle. This 1:10,000 dilution approximates 150 spores/ml in a 2-ml vaccine dose and 300 spores/ml in a 1-ml vaccine dose. This dilution may be too numerous to count.

4.7 Vigorously shake the 1:10,000 dilution by hand 100 times. Using 10-ml pipettes, make a twofold dilution (5 ml 1:10,000 dilution + 5 ml glycerol diluent) in a 20 x 150-mm test tube. This 1:20,000 dilution approximates 75 spores/ml in a 2-ml vaccine dose and 150 spores/ml in a 1-ml vaccine dose.

4.8 Vigorously shake the 1:10,000 dilution by hand 100 times. Using a 5-ml and 10-ml pipette, make a fivefold dilution (2 ml 1:10,000 dilution + 8 ml glycerol diluent) in a 20 x 150-mm test tube. This 1:50,000 dilution approximates 30 spores/ml in a 2-ml vaccine dose and 60 spores/ml in a 1-ml vaccine dose.

4.9 Vigorously shake the 1:50,000 dilution by hand 100 times. Using a 2-ml and 10-ml pipette, make a tenfold dilution (1 ml 1:50,000 dilution + 9 ml glycerol diluent) in a 20 x 150-mm test tube. This 1:500,000 dilution approximates 3 spores/ml in a 2-ml vaccine dose and 6 spores/ml in a 1-ml vaccine dose.

4.10 Vigorously shake each dilution (1:20,000; 1:50,000; 1:500,000) 25 times before the sample is withdrawn. Use a 2-ml or 5-ml glass pipette to add 1 ml of each dilution to each of 3 sterile petri dishes. Pour 1 tube of tryptose agar

into each plate and swirl slowly 10 times in a figure-8 motion to mix. Allow the medium to harden before inverting plates.

4.11 Dispense 1-ml of uninoculated glycerol diluent into 1-2 sterile petri dishes. Pour 1 tube of tryptose agar into each plate and also 1-2 uninoculated sterile petri dishes. Swirl slowly 10 times in a figure-8 motion and allow the medium to harden before inverting plates. These plates serve as negative controls.

4.12 Incubate the plates at 35°-37°C for 24-28 hours.

4.13 Count the colonies and record the results on the worksheets.

5. Calculation of spore counts

5.1 Total the colony counts of the 3 plates made for each dilution and divide by 3. (This is the average number of spores per ml in that dilution.)

5.2 Multiply the average number of spores per ml by the cattle dose. (This is the number of spores per dose of diluted vaccine.)

5.3 Multiply the number of spores per dose of diluted vaccine by the dilution factor. (This is the number of spores per dose of undiluted vaccine.)

6. Interpretation of the spore count test results

6.1 The spore count results are interpreted as described in 9 CFR, Part 113.66. To be eligible for release, each serial and each subserial of Anthrax Spore Vaccine-Nonencapsulated shall have a spore count sufficiently greater than that of the vaccine used in the immunogenicity test. This ensures that when tested within the expiration period, each serial and subserial must have a spore count of at least twice that used in the immunogenicity test but not less than 2 million spores per dose.

7. Reporting of the spore count test results

Report results of the test(s) as described by standard Section operating procedures.

8. References

8.1 Code of Federal Regulations, Title 9, Part 113.66, U.S. Government Printing Office, Washington, DC, 2005.

8.2 History of reagents: *B. anthracis* challenge culture (IRP 137) was obtained from the Central Veterinary Laboratory, Ministry of Agriculture, Weybridge, Surrey, England. It was derived from a strain developed by Max Sterne, from a Pasteur anthrax vaccine strain, by continuous passage in guinea pigs until virulence could not be further enhanced. It was consistently virulent for guinea pigs but not for rabbits, domestic animals, or man.

9. Summary of revisions

This document was revised to clarify the practices currently in use at the Center for Veterinary Biologics and to provide additional detail. While no significant changes were made that impact the outcome of the test, the following changes were made to the document:

- **Part II 4.11** Negative controls have been added.
- References to pipette sizes were added throughout the text for clarification.